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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. 3OVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Reaction of Human Sera with Eubacterium brachy: Isolation and Characterization of an Extracellular Antigen		5. TYPE OF REPORT & PERIOD COVERED Manuscript for Publication
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Jack W. Vincent, William A. Falkler, Jr., Nicholas F. Delessandro, Robert A. Miller and James R. Heath III		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS J. S. Army Institute of Dental Research Walter Reed Army Medical Center Washington, DC 20012		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A, 3M161102BS10, DA 12
11. CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Medical Research & Development Command SGRD-RMS Fort Detrick, Maryland 21701		12. REPORT DATE 26 July 1984
		13. NUMBER OF PAGES Thirty-eight
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

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18. SUPPLEMENTARY NOTES

None

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Eubacterium brachy, Human Antibody Response, High Performance Liquid Chromatography, Pathogenic Mechanisms

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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Reaction of Human Sera

with Eubacterium brachy:

Isolation and Characterization

of an Extracellular Antigen

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Reaction of Human Sera

with <u>Eubacterium</u> <u>brachy</u>:

Isolation and Characterization

of an Extracellular Antigen

ABSTRACT

Recent studies have demonstrated an association of Eubacterium sp. with the subgingival microflora of patients with chronic periodontitis. One species, Eubacterium brachy, was evaluated to determine the possible mechanisms by which this microorganism may contribute to these diseases. Of 167 sera evaluated by double diffusion in agar, 20.8% displayed reactivity with the sonicated preparation of E. brachy. However, when tested by ELISA, all sera displayed some reactivity. Lines of identity were not shared with other species of Eubacterium but were shared with other clinical isolates of E. brachy. The reactive antibody was identified as IgG by immunoelectrophoresis and was found to be capable of complement fixation. An extracellular antigen was identified in the culture supernatant fluid which reacted with antibodies in human sera. This antigen was isolated by methanol precipitation and purified by gel filtration. The monosaccharides and amino acids of this antigen were identified by high performance liquid chromatography. This antigen was shown to have a molecular weight of 170,000 Daltons and to share a line of identity with the sonicated preparation of E. brachy. The possible role of the organism in the immunopathology of periodontal diseases was discussed.

The role of microorganisms in the etiology of periodontal diseases is well established. A morphological description of the maturation of dental plaque in gingivitis was documented by Löe et al. (14) Numerous cultural studies have been undertaken to attempt to correlate various groups of microorganisms with periodontal diseases. Capnocytophaga (18) and Actinobacillus actinomycetemcomitans (27) have been associated with rapid bone loss in juvenile periodontitis. Other studies have implicated the role of Fusobacterium nucleatum in gingivitis (5) and in early (3) and advanced periodontitis (25). A recent study has described the significance of several species of Eubacterium isolated from human periodontitis (10). These species were detected in 35 to 42% of the subgingival plaque samples and comprised from 3 to 57% of the cultivable flora. In a more detailed study designed to determine the frequency distribution of microorganisms in experimental gingivitis (16) various species of Eubacterium were again identified. Several species of Eubacterium were among the microorganisms found to be more numerous in the subgingival flora than in the residual supra-gingival flora from patients with severe periodontitis (17). Furthermore, these same species were more prominent in the subgingival flora of patients with moderate periodontitis than in supragingival plaque or subgingival plaque of periodontally healthy patients (15). E. brachy has also been isolated from a

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pleuropulmonary infection (20).

Extensive characterizations of polysaccharide antigens of *Eubacterium saburreum* have been performed. The antigens of strain L44 (8) were found to be neutral polysaccharides, while strain L49 contained O-acetylated glycero-glacto-heptose (7). Strain L452 produces a polysaccharide composed of trisaccharide repeating units (6), while a dideoxyheptose has been identified as the immunodominant sugar of strain L32 (9). D-glycero-D-glacto-heptose and a 6-deoxyheptose have been isolated from strain 02/725 (23). A periodate-resistant antigen of strain L13 was found to contain a ketohexose as a main sugar constituent (24).

The purposes of this study were to determine and characterize the human antibody response to *Eubacterium brachy* and to isolate and partially characterize the major antigen.

MATERIALS AND METHODS

Cultivation:

Eubacterium brachy (ATCC 33089) (American Type Culture Collection, Rockville, MD), a Gram-positive anaerobic rod, was grown in peptone yeast-extract glucose medium (PYG) supplemented with (per 1,000 ml) $CaCl_2$, 0.2 g; $MgSO_4$, 0.2 g; K_2HPO_4, 1 g; and KH_2PO_4, 1 g (11). Microorganisms were cultured anaerobically in a BBL (Baltimore Biological Laboratory, Cockeysville, MD) GasPak anaerobic jar system at 37°C. The cells were harvested by

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centrifugation at 10,000 X g for 10 min at 4°C, washed 3X in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2, resuspended in PBS as a 1:10 dilution (V-V) of packed whole cells obtained by centrifugation at 2,000 X g for 10 min, and stored at -20°C. This was designated as the whole cell preparation (WC).

Antigen Preparations:

A 1:10 dilution of WC was prepared in 60 mM carbonate buffer (pH 9.6) for use in an enzyme-linked immunosorbent assay (ELISA). Suspensions of WC (3 ml) were mixed 3:1 with 5 μm glass beads (Heat Systems-Ultrasonics Inc., Plainville, NY) and sonicated with sixteen 30 sec bursts in a dry ice-alcohol bath with a Heat Systems Sonicator (Heat Systems-Ultrasonics Inc., Plainville, NY) at a microtip setting of 7. Greater than 95% of the cells were lysed as observed by phase-contrast microscopy. This preparation was designated as the sonicated preparation (SP).

Saturated ammonium sulfate was added to the SP at 22°C until 50% saturation was achieved or the SP was mixed 1:8 in absolute methanol. Both preparations were incubated at 4°C for 18 h. Following centrifugation at 2,000 X g for 10 min, the precipitates were resuspended in PBS. The ammonium sulfate precipitate was dialyzed for three days against repeated changes of PBS and designated ammonium sulfate precipitate (ASP), while the precipitate resulting from methanol treatment was designated methanol precipitate (MP). In addition, the cell free broth supernatant

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fluid was subjected to the same regimen of precipitation and designated broth ammonium sulfate precipitate (BAP) or broth methanol precipitate (BMP).

Double diffusion in agar

The SP was reacted by double diffusion in agar (21) in 1% agarose in PBS with 167 undiluted human serum samples. After identifying reactive sera the ASP, MP, BAP and BMP were subjected to double diffusion in agar with selected reactive sera. All reactions were incubated at 4°C in a humidor and observed after 24 h. The SP of $E.$ brachy was compared with similar preparations of Eubacterium alactolyticum (ATCC 23263), Eubacterium limosum (ATCC 8486), Eubacterium limosum (ATCC 10825), Eubacterium nodatum (ATCC 10825), Eubacterium saburreum (ATCC 33271), and five oral isolates of $E.$ brachy obtained from Virginia Polytechnic Institute (VPI) by double diffusion in agar with a reactive human serum to $E.$ brachy.

Immunoelectrophoresis:

Selected human reactive sera were subjected to immunoelectrophoresis in 1% agarose in borate buffer containing: distilled water, 500 ml; diethyl barbituric acid, 22.4 g; Tris hydroxymethylaminomethane, 44.3 g; calcium lactate, 0.533 g; sodium azide, 1.0 g; and distilled water to 1,000 ml. This was diluted 1:4 with distilled water for use at a final pH of 8.6 (21). Samples were run on a Gelman Semimicro Electrophoresis Chamber

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(Gelman Instrument Co., Ann Arbor, MI) at a voltage of 75 V per slide and a constant amperage of 2 mA per slide. Electrophoresis was continued until the bromophenol tracker dye approached the anodic end of the slide. After electrophoresis, one trough received 100 μl of SP while the other trough received either goat anti-human γ, α or μ heavy chain serum. Immunodiffusion was allowed to progress for 24 h in a humidor at 4°C.

A sample of the SP was subjected to immunoelectrophoresis in the identical manner and subjected to immunodiffusion against two reactive human sera.

Serological Evaluation by ELISA

Human sera (6) previously shown to be reactive with E. brachy by double diffusion in agar and six sera which did not react with E. brachy were subjected to evaluation by a modification of an enzyme-linked immunosorbent assay (ELISA) (19). Briefly, a 200 μl aliquot of a 1:10 dilution of the previously described WC ,reparation in 60 mM carbonate buffer (pH 9.6) was added to each well of a 96-well microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) and incubated at 37°C for 3 h. Peripheral rows were not utilized. The WC preparation was removed and each well was washed 5X with 0.01 M PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO). Each well then received 200 μl of a 1% bovine serum albumin (Grand Island Biological Company, Grand Island, NY) solution in 60 mM carbonate buffer and incubated for

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18 h at 4°C. Following washing 5X with PBS Tween 20, serial twofold dilutions of each serum (100 μl) were added to the appropriate wells and the plates incubated for 30 min at 37°C. The plates were again washed 5X with PBS Tween 20 and received 100 μl of a 1:300 dilution of peroxidase conjugated goat anti-human immunoglobulins γ, α and μ heavy-chain serum (Cappel Laboratories Inc., Cochranville, PA) and the plates were incubated again at 37°C for 30 min. After again washing the plates 5X, 100 μl of an enzyme substrate was added. (This substrate was made up of 1 ml of 1% [wt/v] O-phenylenediamine in absolute methanol combined with 99 ml of distilled water and 0.1 ml of 3% H_2O_2.) The plates were incubated in the dark for 30 min at room temparature, the reactions were stopped by the addition of 50 μl of 8N H_2SO_4, and the intensity of color resulting was determined colorimetrically at 490 nm with an MR 580 Microelisa Auto Reader. (Dynatech Laboratories, Inc., Alexandria, VA). Reactivity was determined as the reciprocal of the highest dilution giving an OD reading 0.1 greater than the serum control. A sample of each serum was adsorbed with E. brachy whole cells prior to being subjected to the ELISA.

Complement Mediated Hemolysis

A 2.5% (V-V) suspension of sheep red blood cells (SRBC) was sensitized with an equal volume of a 1:10 dilution of the SP or BMP and incubated for 30 min at 22°C. Following centrifugation

at 300 X g for 10 min at 4°C, the SRBC were resuspended in PBS as a 2.5% suspension. All sera to be tested were first diluted 1:10 and then adsorbed with an equal volume of 2.5% SRBC for 30 min at 22°C followed by centrifugation at 300 X g for 10 min. The resulting supernatant fluids contained the sera at a 1:20 dilution. These were heat treated at 56°C for 30 min immediately prior to use. Serial twofold dilutions of each adsorbed serum were plated in 96 well Micro Test II culture plates (Becton Dickinson and Co., Oxnard, CA), 50 µl per well. To this was added 25 µl of sensitized SRBC and incubation was allowed to proceed for 1 h at 37°C. Following incubation, 50 µl of guinea pig complement (Whittaker M. A. Bioproducts, Walkersville, MD) was added to each well and the plates were sealed, agitated, and incubated for 1 h at 37°C. Titers were recorded as the reciprocal of the highest dilution demonstrating a 1+ hemolysis.

Identification of an extracellular antigen (ECA)

E. brachy was streaked on PYG agar plates and incubated for 72 h at 37°C. Following incubation, wells were cut in the agar near areas of growth and 15 µl of a reactive human serum was placed in the wells and incubated for 18 h at 4°C. Reactive serum placed in wells far removed from areas of growth served as controls.

Purification of the ECA

The BMP was used for isolation of the ECA by gel filtration

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on a 2.5 cm x 100 cm column packed with degassed Sephacryl S-200 superfine (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS at a flow rate of 1 ml/min. Fractions (5 ml) were collected on an Isco Fraction Collector UA-5 monitored with a type 6 optical unit at 280 nm. A P-3 peristaltic pump was utilized to insure uniformity of flow. Blue dextran (2 X 10^6 Daltons), aldolase (158,000 Daltons), ovalbumin (45,000 Daltons), chymotrypsinogen A (25,000 Daltons), and ribonuclease A (13,700 Daltons) were used as standards. Fractions demonstrating absorbance at 280 nm were tested by double diffusion in agar against a reactive human serum. Those fractions demonstrating a precipitation reaction were pooled, precipitated 1:8 in absolute methanol at 4°C, harvested by centrifugation, resuspended in PBS at the original concentration, and prepared for evaluation by high performance liquid chromatography (HPLC).

Monosaccharide chromatography

A modification of the technique of Alpenfels (1) was used for the identification of monosaccharides as their dansyl hydrazones by HPLC. Briefly, 200 µl of the ECA and selected monosaccharide samples (10 mg/10 ml) were blown to dryness with nitrogen. These samples were then hydrolyzed in 50 µl of 2N HCl at 100°C for 3 h following which all samples were again blown to dryness with nitrogen. To each sample was added 32 µl of water, 20 µl of trichloroacetic acid (0.5 g in 100 ml absolute ethanol) and 40 µl

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of Dansyl-Hydrazine (Sigma, St. Louis, MO) (100 mg in 10 ml of ethanol). All samples were then incubated at 50°C for 50 min following which they were again blown to dryness with nitrogen and resuspended in 1.6 ml of distilled water. A Sep-Pak Ca_{18} cartridge (Waters Associates, Milford, MA) was activated with 2 ml of 100% acetonitrile followed by 1 ml of distilled water and used to remove reaction by-products. Each sample was injected into a cartridge, at which time 2 ml of 10% acetonitrile was used to elute the reaction by-products, followed by an injection of 2 ml of 40% acetonitrile to elute the dansylated monosaccharides. All samples were stored at 4°C and used within 24 h of dansylation.

All tests were performed using a Waters Associates ALC/GPC-244 Liquid Chromatograph equipped with a Model 660 Solvent Programmer, and auxiliary M 6000 A Solvent Delivery System and an Aminco Fluor-Monitor with Spectrum 1012A filter and amplifier. The standards and samples were separated on two μ-Bondapak C_{18} reverse phase columns (Waters Associates, Milford, MA) in series. The isocratic conditions used for each run consisted of a solvent concentration of 22% acetonitrile, Baker HPLC grade in water (V-V) at a flow rate of 2 ml/min with a column temperature of 40°C and a constant chart speed of 30 cm/h. The Aminco multiplier was set at 3.0 and the adjust at medium. The Spectrum attenuation was set at 1 with a cut-off frequency of

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0.02. The excitation wave length setting was 365 nm and the emission cut-off of 480 nm. Following each run, the chromatograph was taken up to 90% acetonitrile, held for 4 min, then returned to isocratic conditions over 2 min and allowed to equilibrate prior to the next run to remove any retained hydrazine from the columns.

Amino acid chromatography

For preparation of samples for analysis of amino acids, 0.5 ml of each sample was mixed with an equal volume of 6 N HCl and incubated for 16 h at 105°C. The samples were blown to dryness with nitrogen. To each dried sample was added 0.3 ml of 0.5 M carbonate buffer (pH 9.0) and 0.3 ml of dansyl chloride (1 mg/ml acetone) and they were incubated for 15 min at 55°C in a water bath. The samples were dried under a stream of nitrogen, reconstituted in 1 ml of absolute methanol and filtered through a 0.45 micron fluropore filter. A 50 µl aliquot was injected into the Waters Chromatograph and was eluted at a constant flow rate of 1.5 ml/min by 0.05 M sodium acetate buffer, pH 4.01, acetonitrile solvent system. The solvent system was programmed to remain at 5% acetonitrile and 95% sodium acetate buffer for 10 min, then increased linearly to 20% acetonitrile and 80% sodium acetate buffer in 15 min followed by a 75 min linear gradient to 40% acetonitrile and 60% sodium acetate buffer. At this point, the compounds of interest had eluted and the system

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was programmed to 100% acetonitrile to remove residual amino acid
and polyamine derivatives.

RESULTS

Double diffusion in agar

Thirty-four of the 167 sera tested (20.8%) displayed a
precipitation line with the SP of E. brachy, as summarized in
Table 1. After identification of reactive sera, several sera
were selected to evaluate the SP, ASP, MP, BAP, and BMP of E.
brachy. The ASP and BMP shared lines of identity with the SP of
E. brachy when evaluated by this method (Figure 1) while the MP
and BAP failed to develop precipitation lines when reacted with
the same human serum.

When the SP of E. alactolyticum, E. limosum, E. nodatum, and
E. saburreum were reacted with this same human serum, no precipi-
tation lines were observed (Figure 2); however, the five oral
isolates from VPI all shared lines of identity with the SP of E.
brachy (Figure 3).

Immunoelectrophoresis

After electrophoresis of a reactive human serum, the class of
antibody reactive with the SP of E. brachy (Figure 4) was deter-
mined to be of the IgG class. When the SP of E. brachy was
subjected to electrophoresis, a single line of precipitation was
observed with a reactive human serum (Figure 5) showing the
presence of a single major antigen in the SP.

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Serological evaluation by ELISA

ELISA results supported the findings observed by double diffusion in agar. Those sera reactive by precipitation demonstrated high titers of activity in the ELISA (Table 2) while those sera not demonstrating precipitation demonstrated much lower activity by ELISA. Prior adsorption of sera resulted in a distinct reduction in the titers obtained.

Complement Mediated Hemolysis

A total of ten sera were tested by complement mediated hemolysis. Of this total, six sera had failed to react with the SP by double diffusion in agar, while four sera had displayed precipitation bands. All ten sera had demonstrated reactivity by ELISA as previously described. The sera nonreactive by double diffusion in agar failed to react by complement fixation, while all of the reactive sera demonstrated hemolysis (1+) at a mean titer of 1:320 (Table 3). These data support the fact that the IgG antibodies are a class known to be capable of complement fixation.

Identification of an ECA

When reactive serum was placed in the wells prepared in the inoculated agar plates, distinct precipitation bands were evident by 18 h (Figure 6). The absence of precipitation bands around the well far removed from the inoculum served as a medium control. These suggested the secretion of an antigenic substance by

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E. brachy which was capable of diffusion through the agar medium.

Purification of the ECA

The results of the gel filtration chromatography are shown in Figure 7. Based on the elution pattern of the various standards from the column, it can be estimated that the approximate molecular weight of the ECA is 170,000 Daltons (fractions 41-44).

Monosaccharide chromatography

When subjected to HPLC analysis for monosaccharide content, the ECA resulted in two elution peaks at 14.6 min and 16.0 min (Figure 8). When compared with the retention times of various monosaccharide standards (Table 4) obtained by identical runs, these two times corresponded to α-D-glucose and D-mannose, respectively. Verification of these findings was obtained by adding a known quantity of either α-D-glucose or D-mannose to the ECA on subsequent runs, thus verifying a spiking of the respective monosaccharide peaks.

Amino acid chromatography

The chromatogram obtained when the ECA was subjected to HPLC analysis for amino acid content demonstrated eight peaks with retention times from 39.42 to 70.10 min (Figure 9). When compared with standards which had been obtained from identical runs (Table 5), seven of these amino acids could be identified. As with the monosaccharide chromatography, each of these seven identifiable peaks was spiked by the addition of the appropriate

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amino acid standards on separate runs, thus verifying the identity of each peak.

DISCUSSION

The presence of a polysaccharide antigen isolated from strains of E. saburreum has been well documented in the literature; however, there appears to be biochemical and antigenic differences between different strains of this microorganism. The results of this study demonstrate the presence of an extracellular antigen produced by the type strain of E. brachy which was antigenically related to other clinical isolates of E. brachy, but not to other species of Eubacterium.

Sera from human subjects, when reacted with SP of E. brachy, demonstrated that 20.8% of those sera displayed reactivity with SP of E. brachy, while, by ELISA, all sera tested displayed some reactivity reflecting the greater sensitivity of this assay. This presence of a naturally occurring precipitating antibody in the serum of human subjects is similar to the finding of Levine and Bush (13); however, major differences are evident in that the previously demonstrated precipitin reacts with various oral bacteria (12). The precipitating antibody we described reacts with the SP of E. brachy but not with any other species of Eubacterium tested.

The finding of an IgG class of antibody reacting with a polysaccharide containing antigen is unusual in that polysac-

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charides usually stimulate IgM. However, this reactivity may be directed toward protein determinants associated with this glyco- protein. The findings of Levine (12) and Skaug (24) also des- cribe similar reactions of IgG antibody reactive to polysac- charide antigens. In the first study, reaction was specific for a D-alanyl ester of glycerol teichoic acid while the latter study demonstrated serum reactive with a periodate-resistant antigen containing ketohexose and glucose obtained by trypsin digestion. Both of these antigens required extensive treatment of whole cells for extraction, whereas the antigen we describe appears to be secreted into the culture medium. The significance of such a substance in the etiology of periodontal diseases appears signi- ficant. The ability of microorganisms to penetrate the gingival sulcular epithelium has not been a consistent finding and appears to occur only in the later stages of disease when obvious micro- ulceration of the epithelium is present. An organism such as E. brachy, which has been isolated from the subgingival plaque of patients with periodontal diseases, appears to possess a mechanism whereby a substance, produced and secreted by this bacterium, may be able to penetrate the sulcular epithelium in the absence of direct microbial invasion. Once present in the connective tissue, this antigen would appear capable of eliciting an immune response by the host, primarily by stimulating the production of IgG precipitating antibodies. If these antibodies

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react with the ECA it is conceivable that immune complexes may form which is suggestive of a type III hypersensitivity response with activation of complement. Acting in this or other mechanisms it would appear that _E_. _brachy_ would play a contributory role in the immunopathology of periodontal diseases. This capability has been demonstrated by the ability of other microbial substances to penetrate an intact junctional epithelium including endotoxin (22), hyaluronidase and collagenase, if preceded by hyaluronidase (2), streptococcal polysaccharide following hyaluronidase (4), and ^{14}C-phenytoin and ^{14}C-albumin (26).

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TABLE 1

Reactivity Of Human Sera With SP Of E. Brachy
As Determined By Double Diffusion In Agar

Patient Population	Number of Patients	Number Positive	% Positive
Healthy Subjects	3	1	33%
Chronic Periodontitis	20	7	30%
Edentulous	13	4	30.7%
Unknown Sera	131	22	16.8%
Total	167	34	20.8%

TABLE 2

ELISA Titers Obtained With SP of E. Brachy And Selected Sera[a]

Reactive Sera	Titer Nonabsorbed Serum	Titer Adsorbed Serum
1	11	8
11	9	6
14	8	4
108	10	5
111	11	7
113	11	8
Nonreactive Sera		
4	6	3
101	4	2
102	4	2
107	3	2
116	2	0
121	5	2

[a]Highest serum dilution resulting in an OD greater than 0.1 when compared with control after transforming the results from Geometric Progressions to Arithmetic Progressions (1:10=1, 1:20=2, 1:40=3, etc.)

TABLE 3

Complement Mediated Hemolysis
Utilizing Six Nonreactive and Four Reactive Sera
as Determined by Double Diffusin in Agar[a]

Nonreactive Sera	Titer[a]
95	_____ [b]
97	_____
107	_____
120	_____
121	_____
122	_____
Reactive Sera	
108	1:640
111	1:160
113	1:320
117	1:320

[a]Highest serum dilution demonstrating 1+ hemolysis

[b]No evidence of hemolysis.

TABLE 4

Retention Times of Monosaccharide Standards
When Subjected to HPLC Analysis

Standard Number	Monosaccharide	Retention Time (Min)
1.	D-galactose	9.20
2.	D-xylose and D-arabinose	10:60
3.	D-ribose	11:60
4.	L-fucose	13.00
5.	α-D-glucose	14.60
6.	D-mannose	16.00
7.	L-rhamnose	23.40

TABLE 5

Retention Times of Amino Acid Standards
When Subjected to HPLC Analysis

Standard Number	Amino Acid	Retention Time (Min)
1.	Serine	39.42
2.	Asparic Acid	41.47
3.	Glutamic Acid	42.55
4.	Threonine	46.70
5.	Glycine	47.55
6.	Alanine	53.42
7.	Proline	70.10
8.	Methionine	71.80
9.	Isoleucine	75.38
10.	Leucine	75.38
11.	Phenylalanine	81.23
12.	Cystine and Cysteine	101.15
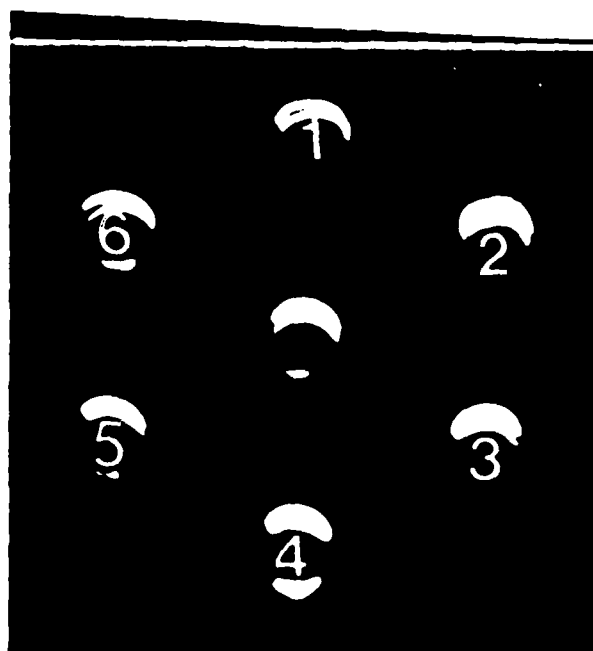
FIGURE 1. Double diffusion in agar showing lines of identity shared by SP(1,4), ASP(2), and BMP(6) but not MP(3) or BAP(5) with a reactive human serum.
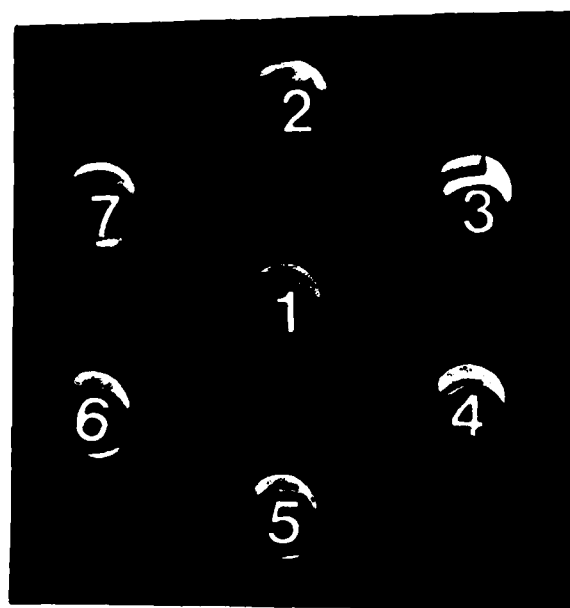
FIGURE 2. Double diffusion in agar utilizing a reactive human serum
(1) to E. brachy (2,4,6) with E. saburreum (3), E. limosum (5), and E.
nodatum (7).
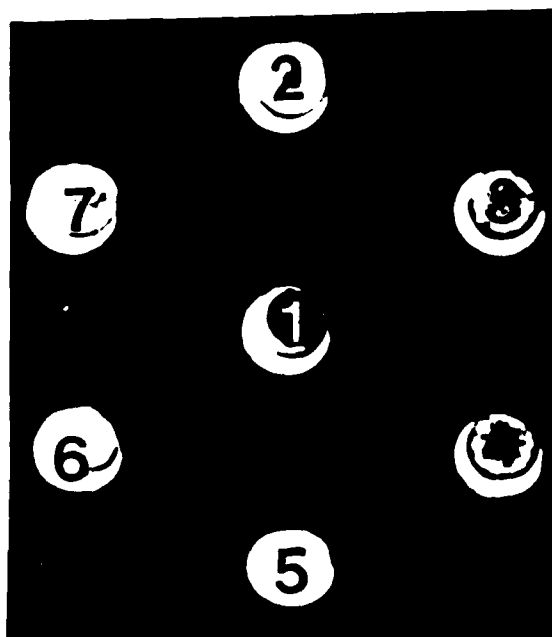
FIGURE 3. Reactivity of a selected human serum (1) with E. brachy ATCC 33089 (2,5) and four clinical isolates of E. brachy (3,4,6,7).
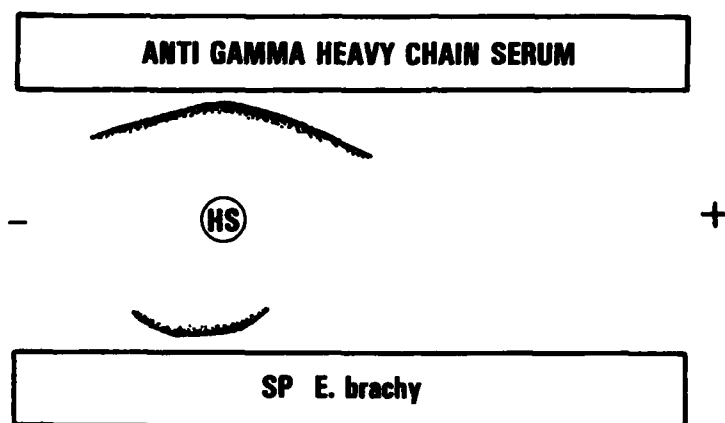
ANTI GAMMA HEAVY CHAIN SERUM

−   (HS)   +

SP E. brachy

FIGURE 4. Representation of immunoelectrophoresis of a reactive human serum (HS) and reactivity with sonicated preparation of E. brachy and goat anti-human gamma chain serum.

REACTIVE HUMAN SERUM

— (SP) +

REACTIVE HUMAN SERUM
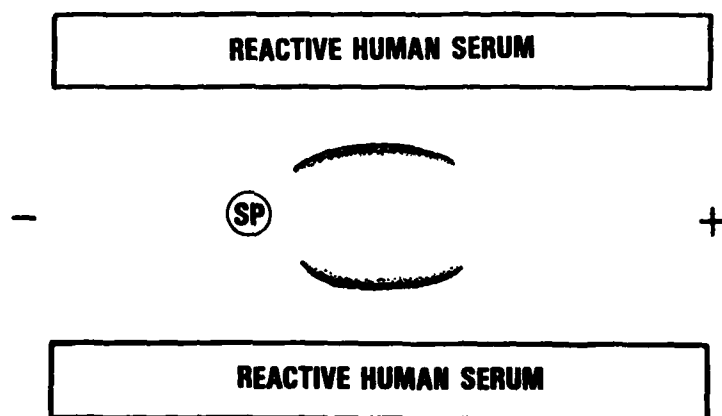
FIGURE 5. Representation of immunoelectrophoresis of the SP and reactivity with two selected reactive human sera.
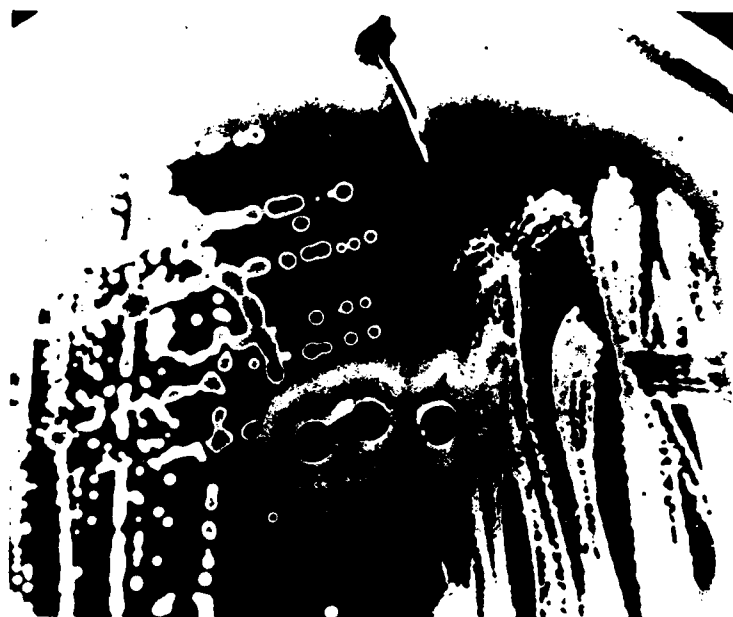
FIGURE 6. Double diffusion in agar utilizing reactive human sera in
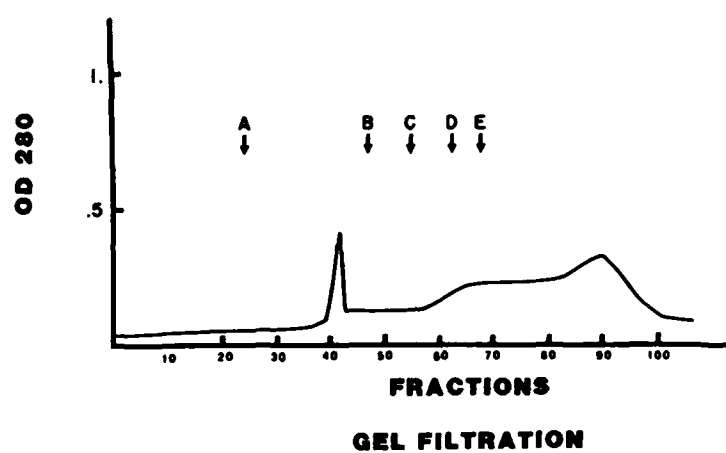wells. Precipitation bands are demonstrating an extracellular
antigen.

GEL FILTRATION

FIGURE 7. Results of gel filtration. Fractions 41-44 contain the purified ECA as determined by immunodiffusion with reactive human sera. Standards (A) blue dextran 2000, (B) aldolase, (C) ovalbumin, (D) chymotrypsinogen A, and (E) ribonuclease A.
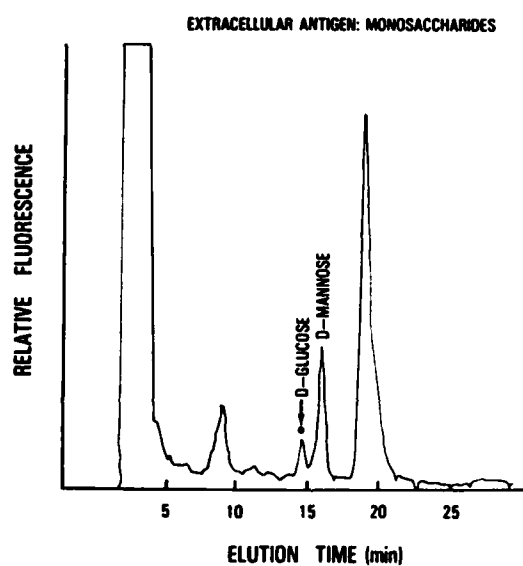
EXTRACELLULAR ANTIGEN: MONOSACCHARIDES

FIGURE 8. HPLC chromatogram showing elution pattern of the two
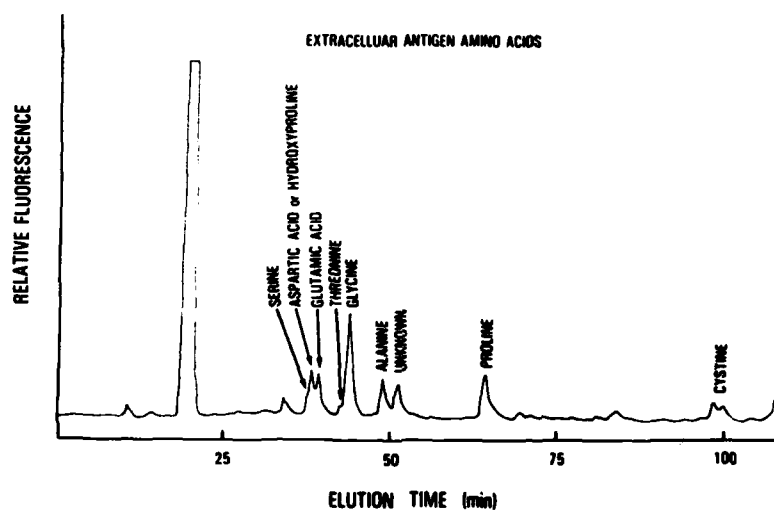monosaccharides contained in the extracellular antigen.

FIGURE 9. HPLC chromatogram of amino acid contained in the extracellular antigen.

END

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DTIC